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Role of NAADP and cADPR in the Induction and Maintenance of Agonist-Evoked Ca^{2+} Spiking in Mouse Pancreatic Acinar Cells

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Summary

Nicotinic acid adenine dinucleotide phosphate (NAADP) and cyclic adenosine diphosphate ribose (cADPR) were first demonstrated to mobilize Ca^{2+} in sea urchin eggs [1, 2]. In the absence of direct measurements of these messengers, pharmacological studies alone have implicated these molecules as intracellular second messengers for specific cell surface receptor agonists [3–7]. We now report that in mouse pancreatic acinar cells, cholecystokinin, but not acetylcholine, evokes rapid and transient increases in NAADP levels in a concentration-dependent manner. In contrast, both cholecystokinin and acetylcholine-mediated production of cADPR followed a very different time course. The rapid and transient production of NAADP evoked by cholecystokinin precedes the onset of the Ca^{2+} signal and is consistent with a role for NAADP in the initiation of the Ca^{2+} response [5, 6]. Continued agonist-evoked Ca^{2+} spiking is maintained by prolonged elevations of cADPR levels through sensitization of Ca^{2+}-induced Ca^{2+}-release channels. This study represents the first direct comparison of NAADP and cADPR measurements, and the profound differences observed in their time courses provide evidence in support of distinct roles of these Ca^{2+}-mobilizing messengers in shaping specific Ca^{2+} signals during agonist stimulation [3–7].

Results and Discussion

A current hypothesis for cholecystokinin signaling in pancreatic acinar cells, based on physiological data, proposes that NAADP acts as an initiator of Ca^{2+} signals by releasing Ca^{2+}, which then triggers Ca^{2+}-induced Ca^{2+} release from $InsP_3$ and ryanodine receptors, thereby amplifying the Ca^{2+} signal [5, 6]. We first investigated the effect of cholecystokinin on latency to initiate Ca^{2+} spiking. The frequency of Ca^{2+} spikes increases with cholecystokinin concentration (Figure 1A). In addition, Ca^{2+} spiking was initiated after a distinct latency (8.7 ± 1.6 to 34.0 ± 2.5 s, n = 10–27) that was inversely dependent on the cholecystokinin concentration (Figures 1A and 1B).

Although the involvement of cADPR in the response to cholecystokinin has been assumed by its sensitivity to the selective antagonist, 8-NH_2-cADPR, there is no direct evidence for an increase in cADPR levels. We next examined the effect of a physiological concentration of cholecystokinin (10 pM) on cADPR production over time by employing a radioreceptor assay based on the sea urchin egg cADPR binding protein [8–11]. The production of cADPR took around 2 min to reach its maximum and, remarkably, remained elevated for several minutes (Figure 2A). This effect was essentially abolished in the presence of cholecystokinin-A receptor antagonist lorglumide (10 μM), suggesting a role for cholecystokinin-A receptors. In addition, cholecystokinin induced an elevation in cADPR levels in a concentration-dependent manner (EC_{50} = 4.6 ± 2.1 pM, n > 3) (Figure 2B).

The neurotransmitter acetylcholine, released from the vagus nerve, acts as an important secretagogue in pancreatic acinar cells by activating muscarinic receptors to also elicit a specific Ca^{2+} signature [3, 5, 12]. Thus, we tested whether acetylcholine stimulates cADPR production. As with cADPR increases in response to cholecystokinin, cADPR levels peaked at 1–2 min by the stimulation of 50 nM acetylcholine, a physiologically relevant concentration, and remained elevated for several minutes (Figure 3A). However, in the presence of the muscarinic acetylcholine receptor antagonist atropine (10 μM), acetylcholine failed to elevate cADPR levels (Figure 3A). Acetylcholine generated cADPR in a concentration-dependent manner (EC_{50} = 6.3 ± 3.4 nM, n > 3) (Figure 3B), consistent with previous measurements [13].

Because NAADP has been proposed to evoke a small localized Ca^{2+} signal before globalization by Ca^{2+}-induced Ca^{2+} release, we reasoned that NAADP should rise within the latent period observed above. Therefore, the early time course of NAADP production in response to cholecystokinin was examined. Figure 4A presents a single experiment demonstrating the effect of a physiological concentration of cholycystokinin (10 pM) on NAADP production over time. After application of cholecystokinin at 10 pM, NAADP levels increased approximately 6-fold on average from a basal level of 0.24 ± 0.08 pmol/mg (Figure 4B, n = 12). The increase in NAADP was maximal at 5–10 s and returned to resting levels after approximately 1 min (Figure 4B). This rapid increase in NAADP level is consistent with the hypothesis that NAADP acts as a trigger to initiate cholecystokinin-evoked Ca^{2+} signals because it becomes elevated within the time frame for latencies for the Ca^{2+} re-

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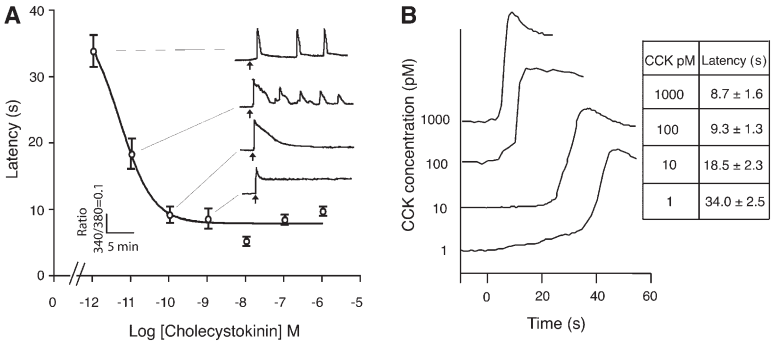

Figure 1. The Effect of Different Concentrations of Cholecystokinin on Ca^{2+} Spiking and Latencies of the Initial Response

(A) The inset shows that at low concentrations, cholecystokinin induced oscillations. The frequency of Ca^{2+} spikes increased with cholecystokinin concentrations. Over a low concentration range, cholecystokinin induced oscillations.

(B) Plot illustrating the effect of cholecystokinin concentration on the initial Ca^{2+} increase over time. Apparent latencies were reduced in a concentration-dependent manner as cholecystokinin concentrations were raised (n = 10–27) as also indicated in the inset table. Data are mean ± SEM.

sponses (Figure 1). Having determined that the increase in NAADP peaked at 5–10 s, we next explored the concentration-response relationship between cholecystokinin and NAADP levels. The resulting concentration-response relationship displayed a shallow slope (Hill coefficient of 0.3) and extended over approximately five orders of magnitude and exhibited an inflection point (Figure 4C). The relationship was best fit to a two-site model, which yielded an EC_{50} of 11 ± 3 pM for the high-affinity site and 830 ± 7 pM for the low-affinity site (Figure 4C, n = 6–9). Lorglumide (10 μM) prevented the increases in NAADP at all cholecystokinin concentrations, confirming the involvement of the cholecystokinin-A receptor.

In contrast to cholecystokinin, a role for NAADP in acetylcholine signaling has been considered unlikely because high inactivating concentrations of NAADP abolish cholecystokinin but not acetylcholine-induced Ca^{2+} spiking [4]. To test the exclusivity of NAADP production for cholecystokinin-A receptor activation, we determined the effect of acetylcholine on NAADP production. A supramaximal acetylcholine concentration (1 μM) at 5 s after application failed to induce a detectable change in NAADP levels (Figure 4D). In the same series of experiments, a physiological concentration of cholecystokinin (10 pM) at 5 s after application induced about a 4-fold elevation of NAADP. These results demonstrate receptor-specific coupling to NAADP production.

Despite intensive studies into the physiological roles of NAADP in Ca^{2+} signaling in pancreatic acinar cells and the common referral to a working model in which NAADP production is linked to cholecystokinin stimulation [5, 6], direct demonstrations of changes in NAADP levels in response to agonists has been lacking. We have now provided the first direct evidence linking cholecystokinin stimulation to NAADP production, thereby demonstrating that it has the hallmarks of a second messenger and also demonstrating a basic tenant of the model. The striking difference in time course between NAADP and cADPR production in response to physiological cholecystokinin concentrations is consistent with our previous "trigger" hypothesis [5, 6] that this agonist, at low physiological concentrations, initially enhances NAADP production, which would induce a localized Ca^{2+} release that then activates cADPR-sensitized ryanodine receptors, allowing the maintenance of Ca^{2+} spiking for the duration of agonist stimulation of pancreatic acinar cells.

It has been reported that the endoplasmic reticulum is continuous in pancreatic acinar cells [14]. However, previous work on pancreatic acinar cells suggests non-uniform intracellular distribution of Ca^{2+}-release channels involved in agonist signaling. Although significant increases of $InsP_3$ have not been detected in response to physiological concentration of cholecystokinin [15], $InsP_3$ receptors are highly concentrated at the apical pole [3, 16], whereas distribution of ryanodine receptors are homogeneously distributed throughout the cell [17, 18]. Accumulating studies suggest that NAADP receptors are more likely concentrated at the apical pole in mouse pancreatic acinar cells because it releases

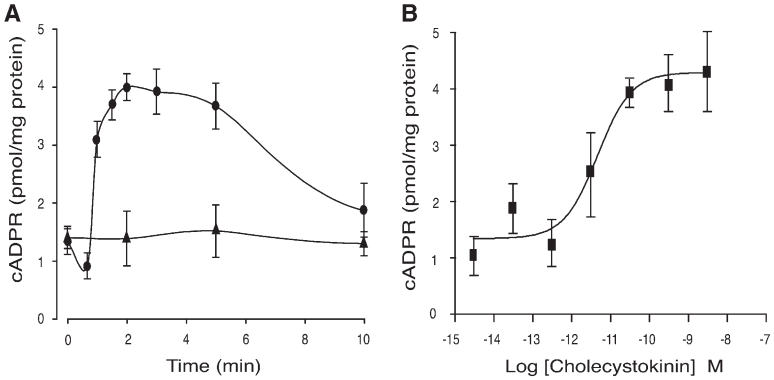

Figure 2. Effect of Cholecystokinin on cADPR Production

(A) The time course of cADPR production (closed circles) was determined in the presence of physiological concentration of cholecystokinin (10 pM). The production of cADPR took around 2 min to reach its maximum, remained elevated for several minutes, and returned to basal levels after around 10 min. The cholecystokinin-A receptor antagonist, lorglumide, inhibited cADPR production (closed triangles).

(B) Cholecystokinin induced increases in cADPR levels (closed squares) in a concentration-dependent manner. The increase was 4-fold with an EC_{50} of 4.6 ± 2.1 pM (n > 3). Data are mean ± SEM.

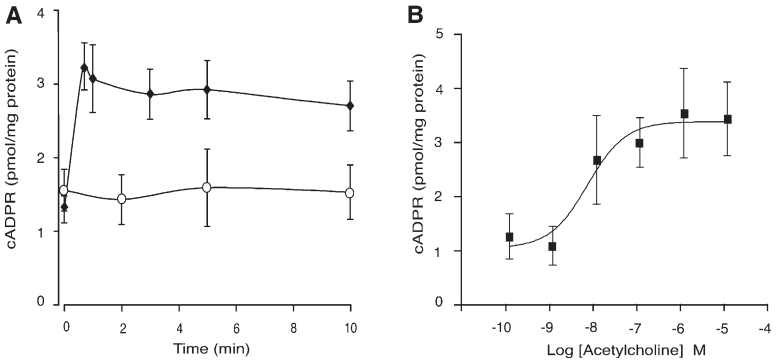

Figure 3. Effect of Acetylcholine on cADPR

(A) A physiological concentration of acetylcholine (50 nM) caused a 3-fold elevation of cADPR levels (closed diamonds), which was prevented by 10 μM atropine (open circles) (n > 3).

(B) Acetylcholine generated cADPR production in a concentration-dependent manner (closed squares) with an EC_{50} of 6.3 ± 3.4 nM (n > 3). Individual data points are the mean ± SEM.

Ca^{2+} at the apical area [7]. Additionally, we have previously suggested that NAADP-sensitive stores in pancreatic acinar cells are predominantly acidic, lysosome-related organelles, possibly secretory granules, which are clustered at the apical pole [7]. The localized increase in Ca^{2+} at the apical pole in response to cholecystokinin requires NAADP to initiate Ca^{2+} release and is also dependent on local recruitment of both $InsP_3$ and ryanodine receptors at the apical zone through positive feedback via Ca^{2+}-induced Ca^{2+}-release mechanisms [5, 6]. As previously reported, however, mitochondria suppress local positive feedback of Ca^{2+} [19], even though $InsP_3$ receptors are highly concentrated at the apical pole [20]. Our new result indicate that the subsequent globalization of the Ca^{2+} signal into basolateral regions occur as cADPR levels slowly rise and sensitize ryanodine receptors there [17] to elevations in Ca^{2+} originating from apical regions while interposed mitochondrial Ca^{2+} barriers are breached [20].

The changes in cADPR and NAADP levels in response to agonists reported here provide a prerequisite for a better understanding of both the coupling mechanisms between receptor activation as well as the enzymology of cellular cADPR and NAADP synthesis. A role for cGMP has been proposed in the regulation of cADPR synthesis by ADP-ribosyl cyclases in pancreatic acinar cells [21], although several other possibilities exist [22]. Furthermore, there are differences in opinion as to whether the principal ADP-ribosyl cyclase involved in pancreatic acinar cell cADPR or NAADP synthesis is CD38 [13, 21]. Although CD38 has been implicated in cADPR synthesis in pancreatic acinar cells [13], the topological paradox of its major location as an ecto-enzyme requires further explanation [23]. In

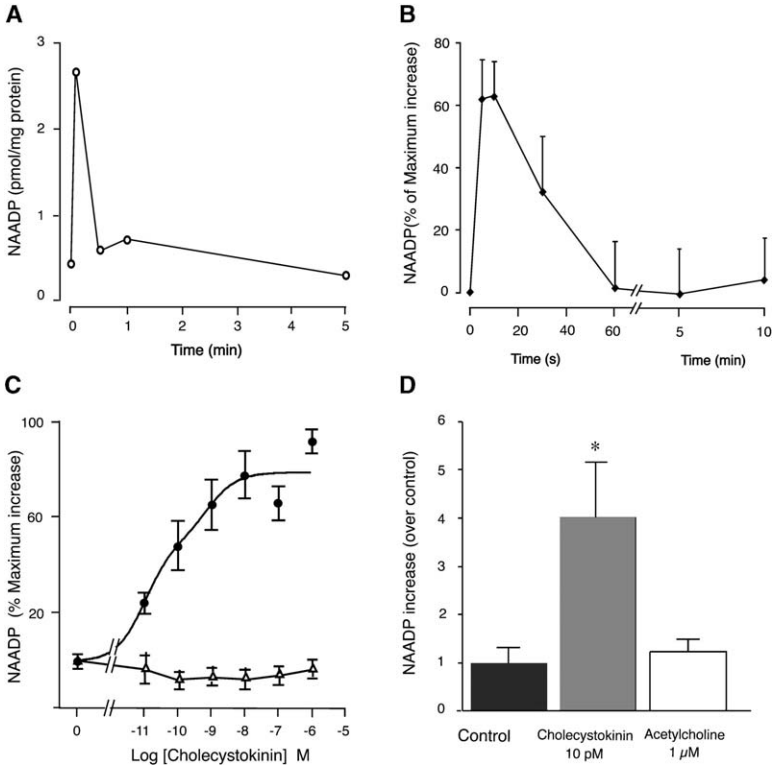

Figure 4. Effects of Cholecystokinin and Acetylcholine on NAADP Production

(A) A single experiment showing the effect of cholecystokinin (10 pM) on the levels of NAADP over time (open circles). This sample showed a rapid increase in endogenous NAADP levels, demonstrating maximum elevation at 5 s after stimulation.

(B) Average results showing the effect of cholecystokinin (10 pM) on the levels of NAADP over time (closed diamonds). The NAADP levels reached a maximum of about 6-fold from a basal level of 0.24 ± 0.08 pmol/mg protein and returned to resting levels in about 60 s (n = 12). Data were normalized to the maximum obtained with each individual time-course experiment.

(C) The absolute values of the basal level and peak are 0.688 ± 0.16 pmol/mg and 6.11 ± 2.03 pmol/mg (n = 6). The data were fitted to the Hill equation with two sites, giving EC_{50}s of 11.0 ± 3.0 pM and 830 ± 6.6 pM (n). Lorglumide, a cholecystokinin-A receptor antagonist, was present at 10 μM (n = 3–6) (open triangles).

(D) A physiological concentration of cholecystokinin (10 pM) caused a significant NAADP increase at 5 s (asterisk, n = 3, $p < 0.05$), whereas a supramaximal acetylcholine (1 μM) did not cause significant elevation of NAADP (n = 3). Data are mean ± SEM.

addition, CD38 has been shown to catalyze the synthesis of NAADP but only in vitro [24]. Our findings and methodologies reported here may help in answering such outstanding questions.

In summary, this study provides direct evidence for a rationale of why multiple Ca^{2+}-mobilizing messengers are produced in response to a given agonist in that their different time courses of synthesis may operate to evoke Ca^{2+} signals with distinct temporal domains.

Experimental Procedures

Isolation of Pancreatic Acinar Cells

Pancreata were excised from male CD1 mice of between 8–10 weeks old and finely chopped before incubation with 200 units/ml collagenase for 15 min at 37°C. The digested tissue was then washed and resuspended to 10 ml with buffer of the following composition: 140 mM NaCl, 4.7 mM KCl, 1.1 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM HEPES, 10 mM glucose (pH 7.2). Protease inhibitor cocktail tablets (Roche Diagnostics), one tablet per 50 ml, were added into this solution before use. The suspension was triturated by pipetting to disrupt the tissue. Supernatant that contained dispersed cells was collected in a new tube and topped up to 15 ml by adding fresh buffer. After centrifugation at 800 × g for 3 min at 4°C, the supernatant was discarded, and the cell pellet was suspended in 6 ml buffer and again triturated in order to further disrupt the tissue. The suspension was centrifuged again as above. The resulting supernatant was discarded, and the cells were kept in buffer supplemented with 1 mM $CaCl_2$ at room temperature on a rocking board for 30 min before use. Prior to use, the cells were centrifuged at 800 × g for 1 min at 4°C. Supernatant was discarded, and the cells were suspended in the solution without added protease inhibitors.

Single Cell Ca^{2+} Imaging

Acinar cells were seeded onto polylysine-coated number 1 glass coverslips and loaded with 1–5 μM fura-2 acetoxymethylester (fura-2/AM) for 30 min at room temperature. Acinar cells were maintained in buffer of the following compositions (in mM): 140 NaCl, 4.7 KCl, 1.1 $MgCl_2$, 1 $CaCl_2$, 10 HEPES, and 10 glucose (pH 7.2). After the loading period, cells were washed and imaged immediately. Coverslips were mounted in a static chamber (Harvard Apparatus) on an inverted Zeiss 35 Axiovert microscope and imaged with a conventional epifluorescence system by using Metafluor software (Universal Imaging). A fraction of the extracellular medium was withdrawn from the chamber by pipette, mixed well with a stock drug solution, and then re-added to the chamber. Cells were excited alternately with 340 and 380 nm light (emission 510 nm), and ratio images of clusters were recorded every 3–4 s with a 12-bit CCD camera (MicroMax; Princeton Instruments, NJ). t = 0 is the time when cholecystokinin was applied into the chamber. The latencies reported here are consistent among our experiments but are overestimates with regard to intrinsic latencies, as previously reported by localized micropipette-application of agonists [25].

Acid Extraction of NAADP and cADPR from a Population of Isolated Cells

Pancreatic acinar cells were prepared in the solution detailed above (with no protease inhibitors). Because the most potent stimuli for secretion of cholecystokinin is the presence of partially digested food in the lumen of the duodenum, food was withdrawn from all the mice used in this study for 6 hr before cell preparations. Agonists were added by pipette into aliquoted population of cells to conform with the way in which the latency experiments were conducted, and the cells were then incubated for the appropriate time at room temperature. Ice-cold 0.75 M $HClO_4$ was added to stop reactions. Sonication (Jencons Vibracell at amplitude 60) was carried out to disrupt the cells, and then the cells were placed on ice for 15 min. The denatured protein was pelleted by centrifugation at 9,000 × g for 10 min and stored at –80°C for later analysis. Supernatant was neutralized with 1 M $KHCO_3$ and vortexed. Centrifugation at 9,000 × g for 10 min was then used to remove the $KClO_4$ precipitate. The resulting supernatant was stored at –80°C for radioreceptor assay analysis. The acid extraction allows 65% recovery of total NAADP or cADPR determined by extracting a sample with a known amount of [^{32}P]NAADP and determining radioactivity recovered and a similar recovery was obtained by using unlabeled NAADP and a Ca^{2+}-releasing bioassay with sea-urchin-egg homogenate [11].

NAADP Radioreceptor Assay

Synthesis of [^{32}P]NAADP was carried out in a two-step reaction as described previously [9]. The [^{32}P]NAADP fraction was then stored at 4°C for use in the assay. The NAADP binding protein from sea urchin (*Lytechius pictus*), which is highly specific for NAADP, was used [8, 10]. Homogenates of sea urchin eggs were prepared as described previously [9]. Binding reactions were carried out for 20 min at room temperature. Reactions were initiated by the addition of 0.5% egg homogenate, 0.25 nM [^{32}P]NAADP, and extract from pancreatic acinar cells. After 20 min, the reaction was terminated by rapid filtration (Brandel cell harvester) through GF/B filters with an ice-cold solution (20 mM HEPES and 500 mM potassium acetate [pH 7.2]). Bound [^{32}P]NAADP was determined by Cerenkov scintillation counting.

cADPR Radioreceptor Assay

Synthesis of [^{32}P]cADPR was performed as previously described [9]. [^{32}P]cADPR binding assay was carried out as for the [^{32}P]NAADP binding assay.

Statistical Analysis

Data are presented as mean ± standard error of the mean. Differences were determined with either Student's t test (two means) or analysis of variance (multiple means). Graphical analysis was performed with Graphpad and Prizm. Significance was taken as $p < 0.05$.

Materials

Radiochemicals were from Amersham Bioscience (UK), and other chemicals were from Sigma except where indicated. Human NAD kinase was kindly provided by Dr. Mathias Ziegler, University of Bergen, Norway.

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